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The Effect of Prostaglandins on Ox Pituitary Content of Adenosine 3′:5′-Cyclic Monophosphate and the Release of Growth Hormone

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1. An assay, based on competition between adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and cyclic [^{3}H]AMP for binding to a rabbit skeletal muscle protein, has been used to measure tissue contents of cyclic AMP. The assay has a sensitivity of 0.05 pmol of cyclic AMP. Cyclic GMP and cyclic CMP have 0.5%, and cyclic IMP 6.5%, of the ability of cyclic AMP to displace cyclic [^{3}H]AMP from binding protein; AMP, ADP and ATP have no effect. 2. By using this method, the cyclic AMP content of ox pituitary slices exposed to prostaglandin was determined; release of growth hormone was measured by radioimmunoassay. 3. Release of growth hormone was increased by 45 min incubation in 1 μM-prostaglandin E_2 in the absence of theophylline, or in 10 nM-prostaglandin E_2, 0.1 μM-prostaglandin A_1 or 1 μM-prostaglandin B_1 in the presence of 0.5 mM-theophylline. 4. Pituitary cyclic AMP content was increased by 10 min incubation in 1 μM-prostaglandin E_2 in the absence of theophylline, or in 0.1 μM-prostaglandin E_2 in the presence of 0.5 mM-theophylline. 5. The maximum increase in cyclic AMP content was observed 10 min, and significant changes in growth hormone release 30 min, after introduction of prostaglandin E_2. 6. The increase in pituitary cyclic AMP content, but not in the rate of release of growth hormone, was observed in the absence of external Ca^{2+}. 7. The stimulation of release of growth hormone by prostaglandin was decreased by preincubation of tissue for 2h in colchicine (100 μM) or cytochalasin B (10 μg/ml). 8. These results support the suggestion that increased release of growth hormone after treatment with prostaglandin is the result of increased tissue cyclic AMP content, and possibly involves a microfilamentous or microtubular protein.

Since the report that release of growth hormone was stimulated *in vitro* by theophylline (Schofield, 1967*b*), an inhibitor of cyclic AMP phosphodiesterase (EC 3.1.4.1), work in several laboratories has shown that increased rates of secretion of growth hormone may be correlated with increased tissue concentrations of cyclic AMP. Thus Steiner *et al.* (1970) found that maximal increases in concentration of tissue cyclic AMP and in the rate of release of growth hormone occurred at the same time after addition of aminophylline (5 mM) to rat pituitary halves, and also that both responses were related to the concentration of aminophylline or of hypothalamic extract in the incubation medium. It has also been established that prostaglandin E_1 increases release of growth hormone by ox pituitary slices (Schofield, 1970), and that prostaglandins E_1 and E_2 increase synthesis and release of growth hormone by rat pituitary halves (Macleod & Lehmeyer, 1970; Hertelendy, 1971). Prostaglandins are capable of increasing cyclic AMP concentrations in rat anterior pituitary halves (Zor *et al.*, 1970).

In the present paper we have investigated whether the rise in tissue cyclic AMP concentration after administration of prostaglandin can explain the increased rates of release of growth hormone. Concentrations of growth hormone were measured by using the specific radioimmunoassay described by Schofield (1967*a*). Cyclic AMP concentrations were measured in pituitary extracts by using competition between cyclic AMP and cyclic [^{3}H]AMP for a binding protein, in an assay similar to that described by Gilman (1970). The binding protein was prepared from rabbit skeletal muscle (Walsh *et al.*, 1968), and the bound-nucleotide–protein complex obtained by filtration through acrylonitrile–polyvinyl chloride co-polymer membranes.

Materials and Methods

Reagents

All unlabelled nucleotides and ox heart phosphodiesterase were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Butyl-PBD scintillator [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.,

and was purified by using charcoal to remove fatty acids (Chen, 1966) and cellulose to remove insulin (Vallance-Owen *et al.*, 1958). Acrylonitrile–polyvinyl chloride filters (Acropor AN-450, 0.45 μm pore size) were purchased from Gelman–Hawksley, Lancing, Sussex, U.K. They were wetted and cut to discs (2.4cm diam.) before use.

Cytochalasin B was obtained from the Development Department, I.C.I. Ltd., Alderley Park, Cheshire, U.K. It was dissolved in dimethyl sulphoxide to a concentration of 5mg/ml, and stored at −15°C.

Prostaglandins were given by Dr. J. E. Pike of Upjohn Ltd., Kalamazoo, Mich., U.S.A. Stock solutions were prepared by dissolving 1mg of prostaglandin in 100μl of ethanol, and the solution was then adjusted to pH6.0–7.5 by addition of 900μl of Na_2CO_3 (1.9mM) and stored at −15°C. The stock solution was diluted before use by addition of 20μl to 1ml of water containing 1mg of albumin. Cyclic [^{3}H]AMP was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. at a specific radioactivity of 2Ci/mmol, or from Schwarz BioResearch (through Cambrian Chemicals Ltd., Croydon, U.K.) at a specific radioactivity of 14Ci/mmol. ^{125}I-labelled growth hormone was prepared by a modification of the procedure described by Greenwood *et al.* (1963). To 2mCi of [^{125}I]iodide (IMS.30; The Radiochemical Centre) were added in sequence 100μl of potassium phosphate (0.5M, pH7.5), 30μl of chloramine-T solution (2mg dissolved in 1ml of potassium phosphate immediately before addition) and 25μl of ox growth hormone (1mg dissolved in 1ml of 0.02M-NaOH). After 45s, 40μl of sodium metabisulphite (22.5mM, dissolved in 0.2M-sodium borate, pH9, containing 0.43M-NaCl), 100μl of NaI (0.15M in borate buffer, pH9) and 100μl of albumin solution (5g of albumin in 500ml of 20mM-sodium borate–43mM-NaCl, pH9) were added. The mixture was then transferred to 8g of Sephadex G-25 equilibrated with albumin solution and contained in a column 25cm×1cm diam., and the ^{125}I-labelled ox growth hormone was eluted with albumin solution. The hormone was stored at −15°C in 0.2ml portions.

Tissue-incubation and -extraction procedures

Incubation of pituitary slices. Pituitary slices were obtained from glands from heifers as described by Schofield (1967*a*), and were incubated at 37°C in 50ml of bicarbonate-buffered salt solution (Krebs & Henseleit, 1932) equilibrated with O_2+CO_2 (95:5) and containing 2.5mM-glucose, sodium glutamate and sodium β-hydroxybutyrate during transfer to the laboratory. They were then transferred to 10ml conical flasks containing 3ml of incubation medium and incubated at 37°C with shaking at 100 cycles/min. Details of the incubation procedures and additions to the medium are given in the tables or in the text. When tissue cyclic AMP concentrations were to be measured after short incubation periods (up to 15min) the flasks were continuously gassed with O_2+CO_2 (95:5). Otherwise they were gassed for 2min before incubation.

At the end of the incubation a sample of the medium was removed for determination of the concentration of growth hormone by using the double-antibody radioimmunoassay described by Schofield (1967*a*). The tissue was then removed, clamped between aluminium blocks cooled in liquid N_2 and stored on solid CO_2 until extraction.

Tissue-extraction procedures. The three methods used for tissue deproteinization were precipitation with $HClO_4$ or trichloroacetic acid, or denaturation by boiling.

(*a*) When $HClO_4$ was used, the frozen tissue was homogenized in 0.35M-$HClO_4$ at 0°C (approx. 75mg of wet tissue/ml) in Kontes conical homogenizers. Precipitated protein was removed by centrifugation at 4°C for 5min in an M.S.E. bench centrifuge at position 10, potassium phosphate (1M) was added to the supernatant to a final concentration of 0.2M, and the pH was adjusted at 0°C to pH 5.5 with 5M-KOH. The $KClO_4$ was then removed by centrifugation at 4°C for 5min.

(*b*) When trichloroacetic acid was used the frozen tissue was homogenized at 0°C in 0.61M-trichloroacetic acid and the protein precipitate again removed by centrifugation. The acid was extracted from the supernatant by eight washes with 5vol. of water-saturated ether, and the ether removed from the aqueous phase by blowing air on to it. The final pH was 5.5–6.0.

(*c*) When the extract was prepared by boiling, frozen tissue was dropped into boiling cyclic-AMP-assay buffer (approx. 75mg of wet tissue/ml) on a boiling-water bath. The tubes were covered to prevent evaporation and boiled for 10min. The tissue was then homogenized and denatured protein removed by centrifugation at 4°C for 5min in a bench centrifuge.

All supernatants were stored at −15°C until the cyclic AMP concentration was measured.

Procedure for measurement of cyclic AMP

Cyclic-AMP-binding protein. Phosphorylase *b* kinase kinase was prepared from rabbit skeletal muscle by ammonium sulphate precipitation as described by Walsh *et al.* (1968). The precipitate was dissolved in 5mM-potassium phosphate, pH7.0, containing 2mM-EDTA, and dialysed against the same buffer; it was not further purified on DEAE-cellulose. The yield from 400g of rabbit muscle was 2.7g of protein, which was stored in small portions at a concentration of 36mg/ml at −15°C.

Cyclic AMP standard solutions. A stock solution of cyclic AMP (1.4mg/ml) was stored in portions at −15°C and standard solutions were obtained by dilution of this stock solution to 50nM (2.5pmol in 50μl). Serial dilutions then gave 20, 10, 5 and 2.5nM.

Incubation. Incubations were carried out at 4°C in polystyrene tubes (LP2; Luckhams Ltd., Burgess Hill, Sussex, U.K.). All reagents were diluted in cyclic AMP-assay buffer, which contained potassium phosphate (0.2M, pH5.5), theophylline (8mM) and 2-mercaptoethanol (6mM).

Filtration. At the end of incubation, 0.6ml of wash medium (0.2M-potassium phosphate, pH5.5) was added to each assay tube at 0°C, and the contents of the tube were immediately transferred to an Acropor membrane and filtered. The membrane was washed with 1ml of wash medium at 0°C, placed in a counting vial and dried at 105°C for 30min, and 5ml of scintillator (5g of butyl-PBD/litre of toluene) added. The radioactivity was determined with a Nuclear–Chicago mark 1 liquid-scintillation spectrometer. Most of the radioactivity remains attached to the filter, which does not dissolve in this scintillator; no attempt was made to correct for efficiency.

Assay conditions. To obtain the required sensitivity, 1pmol of cyclic [^{3}H]AMP was added to each assay tube. Increasing the concentration of binding protein resulted in up to 88% of the added radioactivity being bound (Fig. 1), and a concentration capable of binding 20% of the cyclic [^{3}H]AMP was used in the assay. Subsequently it was found that decreasing the incubation volume from 200 to 100μl decreased the amount of binding protein required and increased the sensitivity of the assay. Binding of cyclic [^{3}H]AMP was essentially complete within 30min at 4°C (Fig. 2), but a reaction time of 90–120min was used to minimize variation within the assay. Addition of reagents (staggered to give each tube the same incubation time) was in the following sequence: (i) standard or unknown cyclic AMP solutions; (ii) binding protein; (iii) cyclic [^{3}H]AMP. The effect of a 2h delay between addition of binding protein (ii) and cyclic [^{3}H]AMP (iii) on the sensitivity of the assay is shown in Fig. 3. Introduction of the delay increased the displacement of radioactivity by 0.12pmol of cyclic AMP from 8.5 to 30.5%, greatly improving the sensitivity of the assay. The assay conditions

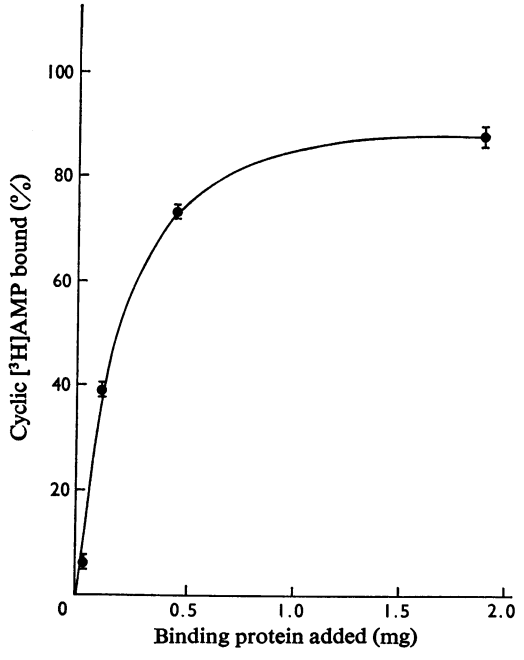

Fig. 1. *Cyclic [^{3}H]AMP recovered on filtration of increasing concentrations of binding protein*

Cyclic [^{3}H]AMP (1pmol; 2Ci/mmol) was incubated for 2h in 200μl of assay medium containing the amount of binding protein indicated, and then filtered by the procedure outlined in the Materials and Methods section.

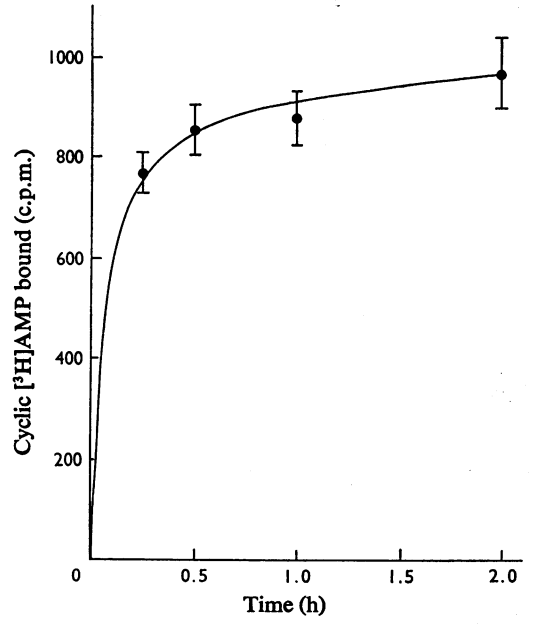

Fig. 2. *Time-course for binding of cyclic [^{3}H]AMP*

Cyclic [^{3}H]AMP (1pmol; 2Ci/mmol) was incubated with 90μg of binding protein in 200μl of assay medium for the indicated times, and filtered by the procedure outlined in the Materials and Methods section.

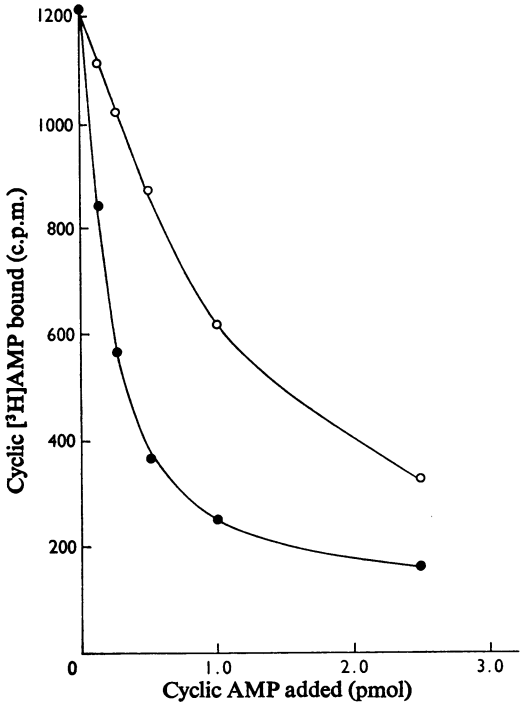

Fig. 3. *Displacement of cyclic [^{3}H]AMP by increasing concentrations of unlabelled cyclic AMP*

Two standard curves, obtained under different conditions, are shown: o, cyclic [^{3}H]AMP (1pmol; 14Ci/mmol), binding protein (30μg) and unlabelled cyclic AMP were incubated in 100μl of assay medium for 90min; •, binding protein (30μg) and unlabelled cyclic AMP were incubated for 120min, after which cyclic [^{3}H]AMP (1pmol) was added and the samples were filtered after 90min.

finally adopted were thus (i) addition of 25μl (30μg) of binding protein to 50μl of unknown or standard cyclic AMP solution, followed by mixing and incubation at 4°C for 2h, and (ii) addition of 25μl of cyclic [^{3}H]AMP (1pmol), followed by mixing, incubation at 4°C for 90min and filtration.

Results

Specificity of cyclic AMP assay

Addition of ATP, ADP or AMP at concentrations likely to be present in tissue extracts had no effect on binding of cyclic [^{3}H]AMP in the assay (Table 1). The cyclic nucleotides guanosine 3′:5′-cyclic monophosphate (cyclic GMP), cytosine 3′:5′-cyclic monophosphate (cyclic CMP) and uridine 3′:5′-cyclic monophosphate (cyclic UMP) all caused some displacement of cyclic [^{3}H]AMP, although they were about 200 times less effective than cyclic AMP. The only cyclic nucleotide to give appreciable displacement was inosine 3′:5′-cyclic monophosphate (cyclic IMP), which was about 15 times less effective than cyclic AMP. The difference in amount of cyclic [^{3}H]AMP bound in the absence of any nucleotide in the two assays shown in Table 1 perhaps requires some comment. The explanation lies in the change in specific radioactivity of cyclic [^{3}H]AMP used, from 2Ci/mmol (The Radiochemical Centre) to 14Ci/mmol (Schwarz BioResearch).

Measurement of cyclic AMP in extracts

In preliminary experiments cyclic AMP solutions prepared in buffer taken through the $HClO_4$ and trichloroacetic acid extraction procedure were compared with standards prepared in the normal assay buffer. The results given in Table 2 show that the extraction procedure with trichloroacetic acid decreased the binding of cyclic [^{3}H]AMP at each concentration of cyclic AMP, perhaps because traces of the acid remained after ether washing. The extraction procedure with $HClO_4$ also decreased binding of cyclic [^{3}H]AMP, but only at concentrations of cyclic AMP below 0.5pmol/100μl. Thus neither $HClO_4$ nor trichloroacetic acid was entirely satisfactory for tissue extraction.

This conclusion was reinforced by experiments in which a large number of tissue extracts obtained by the three different procedures were assayed at two dilutions (Table 3). Twofold dilution of boiled extracts halved the measured cyclic AMP concentration, whereas twofold dilution of $HClO_4$ and trichloroacetic acid extracts decreased the measured cyclic AMP concentration less than expected. This again suggested that in $HClO_4$ and trichloroacetic acid extracts something other than cyclic AMP interfered with binding of cyclic [^{3}H]AMP.

The absence of interference by boiled extracts was confirmed by treatment with phosphodiesterase. Frozen pituitary slices (100mg) were extracted by boiling in 4ml of 200mM-potassium phosphate (pH7.4), containing 5mM-$MgSO_4$, and after centrifugation samples (0.4ml) were incubated at 37°C for 18h with or without ox heart phosphodiesterase (20munits). The reaction was stopped by boiling and cyclic AMP concentrations were determined: the addition of phosphodiesterase decreased the pituitary cyclic AMP content from 0.19$\pm$0.02pmol/mg wet wt. to 0.01$\pm$0.01pmol/mg wet wt., which was not significantly different from zero.

Recovery of cyclic AMP during extraction was determined by adding frozen tissue to extraction medium containing added cyclic AMP (0, 10 or 20nM). After homogenization and neutralization the con-

Table 1. *Specificity of binding-protein assay for cyclic AMP*

The results represent means±S.E.M. for observations in quadruplicate. In the assays in which nucleoside cyclic 3′:5′-monophosphates were added, the volume was 200μl and cyclic [^{3}H]AMP had specific radioactivity 2Ci/mmol. In the assays in which adenine nucleotides were added, the volume was 100μl and cyclic [^{3}H]AMP had a specific radioactivity of 14Ci/mmol.

Nucleotide added (pmol)		Cyclic [^{3}H]AMP bound (c.p.m.)	Amount of cyclic AMP giving equivalent displacement (pmol)
None		396±14	0
Cyclic GMP	5	405±20	0
	20	347± 8	0.1
Cyclic CMP	5	395±13	0
	20	344±20	0.1
Cyclic IMP	2	290± 7	0.3
	10	213±17	0.7
	20	166± 9	1.3
Cyclic UMP	5	407± 6	0
	20	378±16	0.05
None		2385±70	0
AMP	0.5	2421±64	0
	5	2360±106	0
	50	2431±32	0
ADP	0.5	2416±39	0
	5	2476±20	0
	50	2265±46	0
ATP	0.5	2203±70	0
	5	2535±29	0
	50	2385±70	0

centration of cyclic AMP was measured and corrected for cyclic AMP present in the tissue to give recoveries (Table 3). Recoveries were not significantly different from the expected values in boiled extracts, but were only 60–70% in $HClO_4$ extracts.

Thus both the recovery and dilution experiments, and the assay of standard cyclic AMP solutions in extracted buffer, suggested that $HClO_4$ and probably also trichloroacetic acid were not suitable for preparation of extracts. It was observed, however, that identical tissue contents of cyclic AMP were obtained when slices incubated under control conditions were extracted by boiling and in $HClO_4$ (0.12±0.01pmol/mg wet wt. for 12 boiled extracts and 0.13±0.01pmol/mg wet wt. for 27 $HClO_4$ extracts).

Effect of theophylline and prostaglandins on the rate of release of growth hormone

Table 4 presents the effects of prostaglandins A_1, B_1, $F_{2\alpha}$ and E_2 on release of growth hormone during 45min incubation in the presence or the absence of theophylline. The results are the means for several experiments, in each of which slices from one pituitary were exposed to one prostaglandin (at concentrations 0, 10nM, 0.01μM and 1μM, with or without theophylline (0.5mM). Each pituitary yielded 32 slices, of which four were incubated at each condition, and the numbers in parentheses in Table 4 give the total number of slices for all experiments with each prostaglandin. Theophylline (0.5mM) did not stimulate release of growth hormone in any of the experiments; the mean outputs with and without theophylline were 1.31±0.20 and 1.25±0.22μg/h per mg wet wt. ($n = 60$) respectively. The effect of prostaglandin A_1 was not consistent, since at 0.1μM in the presence of theophylline it increased release of growth hormone significantly over the control with theophylline ($P<0.01$), but there was no significant stimulation when the prostaglandin A_1 concentration was increased to 1μM. Prostaglandin B_1 (1μM) in the presence of theophylline stimulated release of growth hormone significantly over controls without theophylline ($P<0.01$), and over controls with theophylline when expressed as a percentage to minimize variation between pituitaries (+44±11%, $P<0.01$). Prostaglandin $F_{2\alpha}$ had no effect on release of growth hormone. Prostaglandin E_2 (1μM) in the absence of theophylline increased the rate of output

of growth hormone significantly over controls without theophylline ($P<0.05$). In the presence of theophylline, however, significant stimulation was obtained at prostaglandin E_2 concentrations over 10nM, and theophylline itself increased release significantly at each prostaglandin concentration. These results, showing that theophylline sensitizes the pituitary to prostaglandin E_2, and that prostaglandin E_2 sensitizes the pituitary to theophylline, are similar to those reported previously with prostaglandin E_1 (Schofield, 1970).

Effect of theophylline and prostaglandin E_2 on the pituitary content of cyclic AMP

Table 5 shows the effect of 10min exposure to prostaglandin E_2 with or without theophylline on the pituitary content of cyclic AMP. The results are means for five pituitaries, two slices from each gland being incubated under each condition. The increases in cyclic AMP observed at 10min were similar to the increases in release of growth hormone at 45min given in Table 4. Thus in the absence of theophylline prostaglandin E_2 increased cyclic AMP concentrations significantly ($P<0.01$) at 1μM, whereas in the presence of theophylline it increased cyclic AMP concentrations at 1 and 0.1μM, but not at 10nM. Theophylline significantly increased tissue cyclic AMP concentrations in the presence of 10nM- and 0.1μM-prostaglandin E_2, although the apparent doubling of cyclic AMP concentration observed at 1μM was not significant. Table 5 also shows that release of growth hormone was not changed after 10min exposure to prostaglandin E_2.

Time-course of cyclic-AMP and growth-hormone responses to prostaglandin E_2 and theophylline

The above experiments appeared to show that changes in tissue cyclic AMP contents could be

Table 2. *Effect of trichloroacetic acid and perchloric acid on the assay of cyclic AMP*

Solutions of cyclic AMP were prepared in cyclic AMP-assay buffer or in the same buffer taken through trichloroacetic acid or $HClO_4$ extraction procedures. The results are means±S.E.M. for the numbers of determinations (each made in quadruplicate) shown in parentheses. Cyclic [^{3}H]AMP had a specific radioactivity of 14Ci/mmol and the assay volume was 100μl.

	Cyclic [^{3}H]AMP bound (% of binding in control buffer in the absence of unlabelled cyclic AMP)		
Added unlabelled cyclic AMP (pmol)	Neutralized trichloroacetic acid	Control	Neutralized $HClO_4$
0	74±6 (2)	100 (12)	79±4 (4)
0.125	58±10 (2)	81±2 (12)	59±7 (4)
0.25	42±7 (2)	61±3 (12)	51±4 (4)
0.5	29±5 (2)	42±2 (12)	39±3 (4)
1.0	18±3 (2)	30±1 (12)	32±3 (4)
2.5	15±2 (2)	17±1 (12)	19±3 (4)

Table 3. *Recovery and dilution experiments on tissue extracts*

'Dilution ratio' is the ratio of the cyclic AMP concentration measured in the tissue extract to the concentration in the same extract measured at half the dilution, and should therefore be 0.5. Both trichloroacetic acid and $HClO_4$ extracts gave dilution ratios significantly greater than 0.5 ($P<0.01$). Recovery of cyclic AMP involved the extraction of frozen tissue in solutions containing 0, 10 or 20nM-cyclic AMP, and measurement of cyclic AMP in 50μl of extract. The results shown were corrected for endogenous cyclic AMP.

		Cyclic AMP recovered (pmol)	
Extraction procedure	Dilution ratio	After 0.5pmol of cyclic AMP	After 1.0pmol of cyclic AMP
Boiling	0.51±0.04 (19)	0.51±0.08 (10)	1.09±0.08 (10)
Trichloroacetic acid	0.60±0.03 (25)	—	—
$HClO_4$	0.62±0.03 (101)	0.36±0.07 (10)	0.61±0.08 (10)

Table 4. *Effect of prostaglandins on release of growth hormone in the presence and the absence of theophylline*

Pituitary slices were incubated for 45min in 3ml of incubation medium containing 2.5mM-glucose, 2.5mM-sodium glutamate and 2.5mM-sodium β-hydroxybutyrate. Slices were distributed so that for one prostaglandin four slices from each pituitary were incubated under each of the eight conditions, and results are mean outputs±S.E.M.; the numbers of slices contributing to the means are given in parentheses. Levels of significance: (*a*) $P<0.01$ for difference from control without theophylline; (*b*) $P<0.01$ for difference from control without theophylline, and for difference from control+theophylline expressed as percentage (+44±11%); (*c*) $P<0.05$ for differences from control without theophylline; (*d*) $P<0.05$ for differences from control+theophylline, and 1nM-prostaglandin without theophylline; (*e*) $P<0.01$ for differences from control+theophylline, and 0.1μM-prostaglandin without theophylline; (*f*) $P<0.01$ for difference from control+theophylline; $P=0.05$ for difference from 1μM-prostaglandin without theophylline.

Prostaglandin	Theophylline (mM)	Concn. of prostaglandin (μM) ...	Rate of growth hormone release (μg/h per mg wet wt.) 0 (control)	0.01	0.1	1	
A_1	—		1.42±0.30	1.90±0.27	1.85±0.26	1.80±0.23	(16)
	0.5		1.23±0.16	1.51±0.20	2.01±0.23 (*a*)	1.49±0.17	
B_1	—		1.21±0.18	1.28±0.19	1.45±0.34	1.98±0.44	(12)
	0.5		1.47±0.23	1.71±0.25	1.85±0.17	2.10±0.24 (*b*)	
$F_{2\alpha}$	—		1.35±0.18	1.69±0.24	1.27±0.12	1.33±0.12	(12)
	0.5		1.37±0.24	1.53±0.24	1.82±0.29	1.76±0.27	
E_2	—		0.95±0.12	1.19±0.13	1.25±0.15	1.58±0.20 (*c*)	(20)
	0.5		1.14±0.14	1.65±0.17 (*d*)	2.10±0.27 (*e*)	2.08±0.16 (*f*)	

observed before changes in the rate of release of growth hormone. The time-course of both responses was therefore measured to see if there was indeed a lag period before the increase in growth hormone release rates. Fig. 4 shows tissue cyclic AMP concentrations at 5min intervals after exposure of pituitary slices to prostaglandin E_2 (1μM) and theophylline (0.5mM). Four pituitary glands were used in these experiments, two slices from each gland being extracted for measurement of cyclic AMP at each time. Tissue cyclic AMP concentrations rose to a maximum 10min after introduction of the prostaglandin, and thereafter decreased, although they were still higher than the controls even after 20min. Fig. 5 shows the time-course of release of growth hormone in response to prostaglandin E_2 (1μM) and theophylline (0.5mM). The results are averages for four slices from each of two pituitaries incubated in the presence or the absence of prostaglandin and theophylline, the medium being sampled at 30min intervals. The rate of release of growth hormone was linear in control medium, and in the presence of prostaglandin and theophylline it became linear after the initial 30min. Although the time-course suggested the possibility of a time-lag before the rate of release of growth hormone responded to prostaglandin E_2, this cannot be established because of the difficulty of detecting small changes over the large basal rate during the initial stages. Thus taking all 10 experiments where output of growth hormone was measured during the first 10min, the mean release in the presence of theophylline and prostaglandin was 0.16±0.02μg/mg wet wt. (25 slices) compared with control release of 0.12±0.02 (25 slices); the difference was not significant ($t=1.34$).

Requirement for Ca^{2+} for the increases in cyclic AMP content and release of growth hormone

Incubation of pituitary slices in Ca^{2+}-free medium containing 0.5mM-ethanedioxybis(ethylamine)tetraacetate (EGTA) for 90min results in a decrease in the Ca^{2+} content of the tissue from 17.0±2.0 to 3.7±0.8mM (Schofield, 1971). The requirement for Ca^{2+} for the prostaglandin effect was therefore tested by incubating slices for four successive 20min periods in normal or Ca^{2+}-free medium containing EGTA, the medium being discarded and fresh warm medium being added at the end of each period before addition of the prostaglandin. Tissue cyclic AMP contents and concentrations of growth hormone in the medium were measured at 10min and 60min after addition of prostaglandin E_2, and the results are presented in Table 6. Incubation in the presence of prostaglandin E_2 for 60min increased release of growth hormone in the presence but not in the absence of Ca^{2+}. The pituitary cyclic AMP content was increased at 10min, and fell during the next 50min, and this rise and fall was not affected by the absence of Ca^{2+}.

Table 5. *Effect of prostaglandin E_2 on pituitary cyclic AMP content and release of growth hormone*

Results are means±S.E.M. for 10 slices (two from each of five pituitary glands) incubated for 10min at each concentration of prostaglandin E_2. Other conditions were as described in Table 4. Levels of significance: (*a*) $P<0.01$ for difference from control without theophylline; (*b*) $P=0.05$ for difference from 1nM-prostaglandin E_2 without theophylline; (*c*) $P<0.01$ for difference from 0.1μM-prostaglandin E_2 without theophylline, and for difference from theophylline control; (*d*) $P<0.01$ for difference from theophylline control.

	Concn. of theophylline (mM)	Concn. of prostaglandin E_2 (μM) ...	0	0.01	0.1	1
Cyclic AMP content (pmol/mg wet wt.)	0		0.13±0.04	0.12±0.03	0.17±0.05	0.70±0.16(*a*)
	0.5		0.22±0.03	0.24±0.05(*b*)	0.36±0.03(*c*)	1.29±0.26(*d*)
Growth hormone released (μg/10min per mg wet wt.)	0		0.12±0.03	0.14±0.03	0.15±0.04	0.13±0.02
	0.5		0.17±0.04	0.15±0.03	0.20±0.03	0.15±0.02

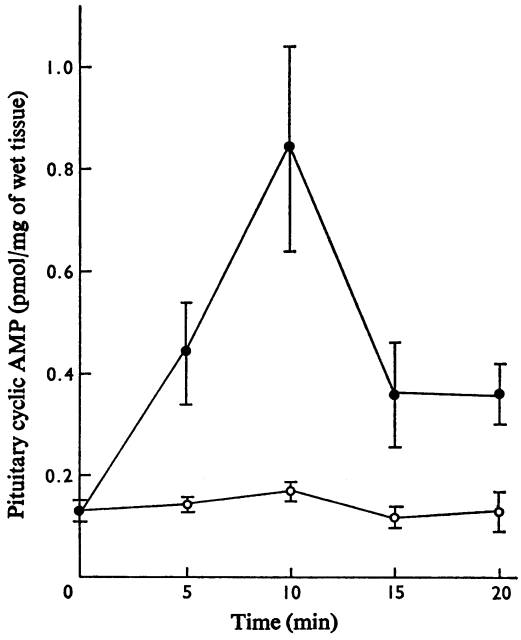

Fig. 4. *Time-course for the change in pituitary cyclic AMP content after addition of prostaglandin E_2*

Pituitary slices were incubated in the presence of 0.5mM-theophylline and 1μM-prostaglandin E_2 (●), or in control medium (○). Vertical bars represent S.E.M. for eight observations. For other details see Table 4.

Effect of colchicine and cytochalasin B on release of growth hormone

Slices obtained from three pituitaries were incubated for 120min in the presence of colchicine (10 or 100μM), the medium being changed at 60min. The slices were then transferred to medium containing colchicine and prostaglandin E_2 (1μM) and incubated for a further 45min. Outputs of growth hormone were measured during this 45min period and the preceding 60min period. There was no change in output as a result of the addition of colchicine during the 60min control period. However, 100μM-colchicine caused a small decrease in the output in the presence of prostaglandin E_2, which was not significant by Student's *t* test but became significant when the variations between different pituitaries were eliminated by using a two-way analysis of variance (Table 7).

In a second series of experiments slices from three pituitaries were incubated in the presence of cytochalasin B (10μg/ml) in place of colchicine. After a 2h exposure to cytochalasin, the stimulation caused by prostaglandin E_2 was strongly inhibited (Table 7).

Discussion

Measurement of concentrations of cyclic AMP

Several methods for measurement of tissue cyclic AMP concentrations have been proposed as alternatives to the procedure of Butcher *et al.* (1965), who used activation of phosphorylase *b*. One such method involves displacement of radioactive cyclic AMP from specific cyclic-AMP-binding proteins (Gilman, 1970; Walton & Garren, 1970; Brown *et al.*, 1971), which in one case were antibodies to cyclic AMP (Steiner *et al.*, 1969). The assay used in these studies was based on that of Gilman (1970), who used bovine skeletal-muscle cyclic-AMP-binding protein, and filtration to separate bound cyclic [^{3}H]AMP from free. A sensitivity better than 0.05pmol of cyclic AMP has been achieved (displacement of 18±1.5% of bound cyclic [^{3}H]AMP, $P<0.001$), compared

with routine sensitivity of 0.2pmol described by Brown *et al.* (1971) and 0.5pmol described by Steiner *et al.* (1969) and Wastila *et al.* (1971). The sensitivity results from the smallness of the incubation volumes used (100μl), and from the 2h preincubation of unlabelled cyclic AMP with binding protein before addition of cyclic [^{3}H]AMP. This procedure, originally used by Hales & Randle (1963) for assay of insulin, improves the sensitivity because exchange of unlabelled and ^{3}H-labelled cyclic AMP is very slow and cyclic [^{3}H]AMP can therefore bind only on sites not containing cyclic AMP.

The specificity of the assay was similar to that described for other assays for cyclic-AMP-binding protein, in that only cyclic IMP of the nucleotides tested showed appreciable displacement of cyclic [^{3}H]AMP. Cyclic IMP is made by the action of nitrous acid on cyclic AMP, but it is unlikely that contamination by cyclic AMP could reach 6.5% by wt. In all probability cyclic IMP can bind to the cyclic-AMP-binding site of the protein. The possibility that the extraction procedure might alter the apparent concentration of cyclic AMP also becomes important, since tissue extract constitutes half the assay volume of 100μl. It would appear that extraction by boiling is the method of choice, since it avoids the interference found with perchloric acid and trichloroacetic acid extracts. Neither Gilman (1970) nor Brown *et al.* (1971) reported interference by perchloric acid or trichloroacetic acid extracts in assays with cyclic-AMP-binding protein, whereas Wastila *et al.* (1971) found that perchloric acid extracts showed inhibition in their protein-phosphorylation assays.

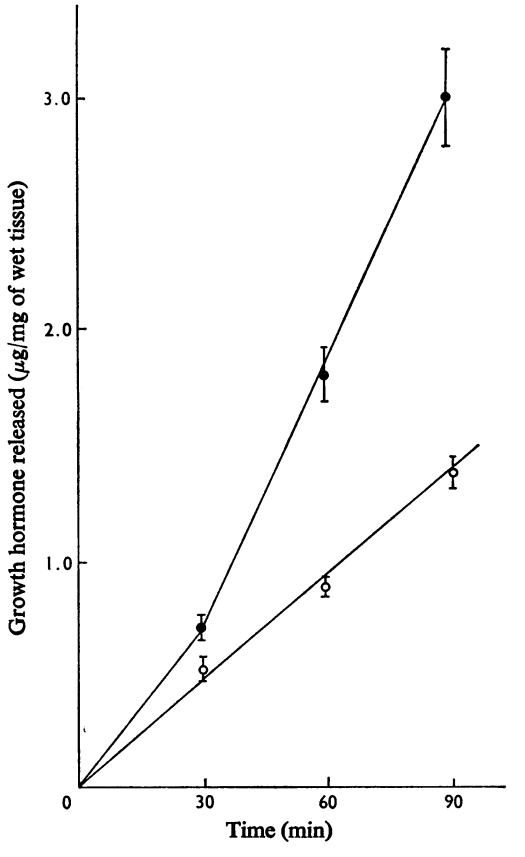

Fig. 5. *Time-course for the change in release of pituitary growth hormone after addition of prostaglandin E_2*

Pituitary slices were incubated in the presence of 0.5mM-theophylline and 1μM-prostaglandin E_2 (●), or in control medium (○). Vertical bars represent S.E.M. for eight observations. For other details see Table 4.

The mean cyclic AMP content of pituitary slices incubated under control conditions was 0.13±0.01 ($n = 39$) pmol/mg wet wt. of tissue, considerably below the concentration of 4–6pmol/mg wet wt. obtained by Zor *et al.* (1970), and below the value of 0.32±0.02pmol/mg wet wt. obtained by Steiner *et al.* (1970) for rat slices incubated *in vitro* for 60min. Assuming an intracellular water content of 40% wet wt. (Schofield, 1971), the concentration of cyclic AMP in heifer pituitary slices may be calculated to be 0.3μM. This is three times the dissociation constant of the cyclic-AMP-binding protein present in pituitary homogenates (0.09μM; E. N. Cole & J. G. Schofield, unpublished work).

Cyclic AMP and release of growth hormone

The results presented in this paper show that prostaglandin E_2 (1μM) can stimulate release of growth hormone from heifer pituitary slices, as can prostaglandin E_1 (Schofield, 1970). The specificity for prostaglandins in the heifer is the same as that described in the rat by Macleod & Lehmeyer (1970). Whether these responses *in vitro* reflect a physiological role of prostaglandins in controlling secretion of growth hormone *in vivo* is not known. However, Ito *et al.* (1971) have shown that prostaglandin E_1 (3μg/kg), infused into humans over 30min, causes an increase in plasma growth-hormone concentration, which is maximal 30–60min after the end of infusion and occurs without any fall in the concentration of blood glucose. If prostaglandins do have a physiological role in secretion of growth hormone, the situation would be complicated by an apparent lack of hormone specificity; prostaglandin E_1 stimulates release of adrenocorticotrophic hormone (Peng *et al.*, 1969), and although it had no effect on release of

Table 6. *Prostaglandin E_2-induced changes in pituitary cyclic AMP content and release of growth hormone in the absence of Ca^{2+}*

Results are means±S.E.M. for six slices obtained from two pituitary glands incubated for each time under each condition. Slices were preincubated for 80min in 3ml of normal or Ca^{2+}-free medium containing 0.5mM-EGTA, 2.5mM-glucose, 2.5mM-sodium glutamate and 2.5mM-sodium β-hydroxybutyrate, and then transferred to 3ml of medium with or without prostaglandin.

Incubation conditions		Growth hormone release (μg/mg wet wt.)		Cyclic AMP content (pmol/mg wet wt.)	
Ca^{2+}	Prostaglandin E_2 (1μM)	10min	60min	10min	60min
+	−	0.10±0.04	0.23±0.02	0.14±0.02	0.06±0.02
+	+	0.10±0.01	0.98±0.15	1.45±0.16	0.27±0.06
−	−	0.08±0.01	0.21±0.05	0.09±0.01	0.07±0.02
−	+	0.08±0.01	0.20±0.01	1.25±0.10	0.22±0.08

Table 7. *Effect of inhibitors on prostaglandin E_2 stimulation of release of growth hormone*

Results are means±S.E.M. for 24 slices obtained from three pituitaries. The slices were preincubated in the presence of inhibitor for 120min, and then exposed to prostaglandin E_2 in the presence of the inhibitor for 45min. Other conditions were as given in Table 4, except that glucose was the sole substrate.

	Growth hormone released (μg/h per mg wet wt.)	
	Control period	With prostaglandin E_2 (1μM)
Control	0.94±0.06	2.73±0.23
Colchicine (10μM)	1.03±0.06	2.59±0.25
Colchicine (100μM)	0.93±0.07	2.14±0.22*
Control	0.62±0.06	3.14±0.34
Cytochalasin (10μg/ml)	0.70±0.07	1.34±0.09†

* $P<0.05$ for difference from control with prostaglandin E_2, by using two-way analysis of variance.
† $P<0.01$ for difference from control with prostaglandin E_2.

luteinizing hormone (Zor *et al.*, 1970) the measurements were made at 20min, when the response might not be apparent.

A close correlation was observed between increased pituitary cyclic AMP contents and release of growth hormone. Thus prostaglandin E_2 at 1μM increased both release of growth hormone and cyclic AMP content, whereas lower concentrations had no effect. Theophylline was used to find whether it potentiated the effects of prostaglandin E_2, and the concentration of 0.5mM was chosen because at this concentration no stimulation of release of growth hormone by theophylline was observed. This contrasts with the results of Ewart & Taylor (1971), who observed stimulation of release of rat growth hormone by 0.5mM-theophylline *in vitro*. In the presence of theophylline at 0.5mM, the concentration of prostaglandin E_2 required to stimulate release of growth hormone was decreased to 10nM. However, significant increases in cyclic AMP content were observed only at 0.1μM-prostaglandin E_2. Whether this difference represents a separation of the two responses, or is caused by the different times of exposure to prostaglandin E_2 (45min for release of growth hormone and 10min for cyclic AMP content) is not clear. The decrease in the effective concentration of prostaglandin E_2 would be consistent with effects of theophylline on phosphodiesterase and of prostaglandin E_2 on adenylate cyclase, although other possibilities such as inhibition of prostaglandin destruction by theophylline cannot be ruled out.

The maximum increase in tissue cyclic AMP content was observed 10min after introduction of prostaglandin E_2, at which time no significant change in release of growth hormone could be detected. This is consistent with the hypothesis that increased release of hormone is a consequence of increased pituitary cyclic AMP content. It should not be assumed that there is a lag before the rate of output of growth hormone is increased, because of the diffi-

culty in measuring small changes in a relatively large parameter immediately after addition of medium containing prostaglandin E_2 to pituitary slices. Moreover, Hertelendy (1971) found a significant increase in release of growth hormone 15min after addition of prostaglandin E_2 to rat pituitary slices. It should be noted that Steiner *et al.* (1970) found a maximum cyclic AMP concentration 30min after addition of hypothalamic extracts. A second point to emerge from the time-course studies was the fall in tissue cyclic AMP concentrations after long exposure to prostaglandin E_2. Rasmussen (1970) has suggested that decreases in cyclic AMP could be due to feedback inhibition by Ca^{2+} on adenylate cyclase. However, after the addition of prostaglandin E_2 to the pituitary in the presence and absence of Ca^{2+}, cyclic AMP concentrations increased identically at 10min and fell identically at 60min, which is difficult to reconcile with the hypothesis of feedback inhibition by Ca^{2+}.

That the increase in tissue cyclic AMP concentration can be dissociated from stimulation of release of growth hormone is apparent from the observation that the prostaglandin-induced increase in release of growth hormone is Ca^{2+}-dependent, whereas the rise in cyclic AMP concentration is not. Steiner *et al.* (1970) showed that hypothalamic extracts can increase cyclic AMP concentrations, but not release of thyrotropic hormone or growth hormone, in the absence of Ca^{2+}, again pointing to the existence of a Ca^{2+}-requiring step that determines whether cyclic AMP can alter the rate of release of hormone. Similar dissociation of cyclic AMP and release of growth hormone was demonstrated by Hertelendy *et al.* (1971), who showed that valinomycin alone increased cyclic AMP content but not hormone release, although in the presence of theophylline it blocked the increase in release of growth hormone without changing cyclic AMP concentrations.

One possible explanation of these results, namely that cyclic AMP increases protein synthesis and hence release of hormone, is precluded by the observations of Macleod & Lehmeyer (1970), who showed that prostaglandin increased release in the absence of protein synthesis. A second possible explanation was suggested by Goodman *et al.* (1970), who found that cyclic AMP increased phosphorylation of neurotubular protein in rat cerebral cortex and proposed that phosphorylation of this protein structure was the first step in neurosecretion. There is now evidence for the involvement of colchicine-sensitive microtubular structures in the secretion of insulin from the pancreatic islets (Lacy *et al.*, 1968; Malaisse *et al.*, 1971) and in secretion of catecholamines from perfused adrenals (Poisner & Bernstein, 1971). Secretion of iodine and colloid-droplet endocytosis in the thyroid are sensitive to colchicine (Williams & Wolff, 1970), and to cytochalasin B (Williams & Wolff, 1971), which is thought to disrupt microfilamentous contractile machinery (Wessels *et al.*, 1971). Thus the demonstration that the stimulation of release of growth hormone by prostaglandin E_2 is also colchicine- and cytochalasin-sensitive suggests an involvement of microfilamentous proteins in secretion of growth hormone, and would be consistent with the hypothesis that cyclic AMP acts at that point in the secretory process. However, Ewart & Taylor (1971) have reported that colchicine does not inhibit release of rat growth hormone *in vitro*, and it remains to be established that cytochalasin B acts specifically on microfilaments.

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